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Principal Author: Mark A. Eiteman

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Name and Address of Submitting Organization:

University of Georgia
Faculty of Engineering
c/o Mark A. Eiteman, Ph.D.
Driftmier Engineering Center
Athens, GA 30602
(706) 542-0833
eiteman@engr.uga.edu

DISCLAIMER:

ABSTRACT:

This report describes results toward developing a process to sequester CO_2 centered on the enzyme pyruvate carboxylase. The process involves the use of bacteria to convert CO_2 and glucose as a co-substrate and generates succinic acid as a commodity chemical product. The phases of research have included strain development and process development. Though we continue to work on one important component of strain development, the research has principally focused on process development.

In the previous year we constructed several strains which would serve as templates for the CO_2 sequestration, including the knock-out of genes involved in the formation of undesirable by-products. This project period the focus has been on the integration of the pyruvate carboxylase gene (*pyc*) onto the *E. coli* chromosome. This has proven to be a difficult task because of relatively low expression of the gene and resulting low enzyme activity when only one copy of the gene is present on the chromosome. Several molecular biology techniques have been applied, with some success, to improve the level of protein activity as described herein.

Progress in process development has come as a result of conducting numerous fermentation experiments to select optimal conditions for CO_2 sequestration. This process-related research has progressed in four areas. First, we have clarified the range of pH which results in the optimal rate of sequestration. Second, we have determined how the counterion used to control the pH affects the sequestration rate. Third, we have determined how CO_2 gas phase composition impacts sequestration rate. Finally, we have made progress in determining the affect of several potential gaseous impurities on CO_2 sequestration; in particular we have completed a study using NO_2. Although the results provide significant guidance as to process conditions for CO_2 sequestration and succinate production, in some cases we do not yet understand the underlying mechanism or reason for the observation. Also, process development has used the “baseline” organism in the absence of the pyruvate carboxylase gene. In some cases the conclusions regarding the process may change when the “final” strain is used which incorporates the key CO2 sequestration technology.

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INTRODUCTION:

Most research on the microbial sequestration of CO_2 has centered on *bacteria* and *archaea* (Atom 2002, Shively et al. 1998) in liquid suspension reactor systems (Kodama 1996) or microalgal systems (Brown 1996, Watanabe and Hall 1996). Many of these microbial species require a photosynthetic reaction to generate ATP for subsequent CO_2 fixation, which severely limits their application for CO_2 sequestration due to scale-up problems, including the requirement for an extremely large reactor size (Zhang et al. 2002). In addition, many of the *bacteria* and *archaea* proposed for CO_2 fixation have fastidious growth requirements, and have unacceptably low product yields and formation rates, both of which essentially eliminate industrial applications (Atom 2002, Shively et al. 1998). CO_2 is a gaseous substrate, and little research has centered on advanced reactor design configurations that significantly improve CO_2 utilization and continuously generate products of interest (Lamare and Legoy, 1993). In fact, a review of the literature shows no reports on the use of bacterial systems for CO_2 fixation in bioreactors; most of the research has centered on the use of microalgal systems that require light/dark cycles and reactors with large footprints (Brown 1996, Wantanabe and Hall 1996, Otsuki 2001). These microalgal reactors typically have extremely slow substrate consumption rates. For example, Otsuki (2001) reports a CO_2 utilization rate of 50 $g/m^2/day$ for a photobioreactor with a surface area to volume ratio of 6.2 m^2/m^3 or a volumetric rate of only 13 mg CO_2/L/hr. This number is the single most important parameter in assessing the viability of a biological CO_2 sequestration process. Substrate consumption and product formation rates are generally more than 100-fold greater in commercially relevant microbial based systems.

The general approach used in this project involves the biological incorporation of CO_2 into the backbone of another inexpensive organic compound to generate a C_{X+1} compound. Promising examples of this strategy include using the enzymes malate oxidoreductases (EC 1.1.1.39, EC 1.1.1.83, etc.) and pyruvate carboxylase (EC 6.4.1.1). Malate oxidoreductases are enzymes which convert pyruvate (C_3) into malate (C_4), while pyruvate carboxylase converts pyruvate (C_3) into oxaloacetate (C_4). It must be understood that a microbial process which relies on a CO_2-fixing enzyme will use a co-substrate biochemically "upstream" of the CO_2-conversion step, while the ultimate product will be a compound biochemically "downstream" of the CO_2-conversion step. Thus, in practice pyruvate carboxylase could convert glycerol or glucose (upstream of CO_2 fixation) into succinic acid (downstream of the CO_2 fixation step).

The chemical product that will be the focus of this project is succinic acid. Cost analysis suggests that commercialization of succinic acid production by a biological route is feasible with improvements in strain and process design (Schilling 1995), and current economic models do not include benefits derived from carbon sequestration. Succinic acid would be used as a chemical feedstock for industrial chemicals such as polymers. As the cost for the chemical route increases in the coming years while improvements in the biological process are attained, a biological route will likely become the preferred route. The approach used in this research will furthermore be quite applicable to other biological processes which sequester CO_2, and we hope that other promising routes involving the use of CO_2 directly in the synthesis of organic C_1 compounds may be more fully developed, such as using formate oxidoreductases (EC 1.2.1.2 or EC 1.2.1.43) to generate formic acid or using urea amidohydrolase (EC 3.5.1.5) to generate urea.

Our research group has many years of experience developing microbial processes for the production of succinic acid and other biochemicals. We have previously demonstrated that we can achieve succinic acid production at near theoretical yields and demonstrated the use of hydrogen gas as an example reducing agent to increase yield from glucose (Vemuri et al. 2002a, 2002b). More importantly, we estimate that the rate of CO_2 utilization by these fermentation routes would be about 45 kg/m^3day (16 metric tons/m^3year), about 150 times greater than the CO_2 utilization reported by Otsuki (2001) in a photobioreactor. This estimate is based on our projection of a 5 g/Lh volumetric succinate productivity in a bioreactor, a rate readily attainable by cell concentration step prior to the bioconversion step. In other words, a 1 m^3 vessel would consume 45 kg of CO_2 and 92 kg glucose per day to generate about 120 kg of succinic acid. (In practice a commodity chemical like this would be produced at the 1000 m^3 scale at one site.)

There has been limited research on the use of CO_2 as a substrate in high density microbial fermentations and advanced reactor designs to improve CO_2 utilization. Fortunately, there has been research in the area of synthesis gas fermentation processes. The composition of synthesis gas varies, but is composed primarily of CO and H_2, both poorly water-soluble substrates. The limited solubility of these gaseous substrates has led to the development of trickle-bed, airlift, and microbubble sparged reactors to enhance mass transfer and subsequently increase the rates of product formation (Bredwell 1999, Wolfrum and Watt 2002). In addition to mass transfer limitations, synthesis gas fermentations are limited by low product yields and concentrations, and by low rates of product formation primarily due to low cell densities (Worden 1997).

The bacteria that produce succinic acid from CO_2 are *Escherichia coli*, the name of the specific strain is AFP111/*pyc*, and it is well characterized genetically and biochemically. Our technology centers on incorporating pyruvate carboxylase enzyme activity into *E. coli* by overexpressing the *pyc* gene that this organism does not normally have. This enzyme uses biotin very effectively and specifically to fix CO_2 onto an organic backbone. The general process involves growing the cells aerobically to a high cell density, and then subjecting them to a prolonged CO_2-rich anaerobic non-growth phase for succinic acid production. Once generated, the cells can remain productive for many cycles. The current barriers in the proposed process for the implementation of this technology include: 1) strain development, 2) process development and 3) reactor design. In the area of strain development, the needs are to knockout genes which lead to by-product formation, overproduce glucose uptake genes, and incorporate pyruvate carboxylase onto the chromosome of *Escherichia coli*. In the area of process development, operating parameters must be optimized such as pH, and the effect of other substances in the gas stream must be assessed. In the area of reactor design, CO_2 mass transfer resistance must be quantified and lead to complete CO_2 utilization. Ultimately, the process must produce succinic acid at high rates and yields. While large reductions in CO_2 levels are not anticipated using such a single process, the technology does provide a niche method for CO_2 transformation into commercial products, may lead to analogous technologies using other biological approaches, and is part of DOE's vision or roadmap for CO_2 sequestration (Creutz and Fujita 2000).

EXECUTIVE SUMMARY:

This report describes results toward developing a process to sequester CO_2 centered on the enzyme pyruvate carboxylase. The process involves the use of bacteria to convert CO_2 and glucose as a co-substrate and generates succinic acid as a commodity chemical product. This research has focused on strain development and on process development.

The primary goal regarding strain development has been to incorporate pyruvate carboxylase into the *E. coli* chromosome. We have previously been successful in integrating the *pyc* gene into the chromosome. However, no pyruvate carboxylase enzyme activity was detected. A variety of molecular biology approaches have been pursued to increase this activity, all related to increasing the strength of the biological promoter. The large size of the *pyc* gene have furthermore made standard protocols problematic, and therefore new procedures have also had to be developed during the course of the research.

We also had several goals in the development of a process. During the last project period we concluded that CO_2 sequestration was not affected by pH in a complex medium. However, in the preferred defined medium, we this year show that pH has a significant impact on sequestration. Based on rate of sequestration as well as rate of base consumption, the optimal pH was found to be about 6.4.

A second goal was to select the optimal base to control the pH during the process. The selection of base is important, and calcium bases such as $Ca(OH)_2$ give the best results. At least part of this effect can be explained by the comparative insolubility of calcium succinate compared with monovalent cations such as sodium or potassium.

A third goal was to quantify the effect of CO_2 concentration in the gas phase on the rate of CO_2 sequestration. The relationship between these factors is linear, with sequestration rate being in proportion to gas phase concentration. Consideration of the chemical and biological kinetics of the systems makes it unclear why this would be the result. The result does suggest, however, than an additional increase in sequestration rate could be attained by pressurizing the system. We do not have the facilities to test this prediction.

Finally, we have begun to determine the effect of gas phase impurities on the sequestration rate. Initial studies using NO_2 have surprisingly indicated that this gas enhances the rate of sequestration, at least when present up to a concentration of 200 ppm. This result demonstrates a robustness for the process in handling real flue gas streams with minimal pre-treatment necessary. Also, this result is worth pursuing because it suggests some underlying phenomenon which improves this sequestration process.

EXPERIMENTAL:

For all experiments, the control strain was *Escherichia coli* AFP111 [ATCC 202021, F+λ-*rpoS396*(Am) *rph*-1 *ldhA*::kan Δ(*pflAB*::cam)] (Donnelly et al., 1998; Chatterjee et al. 2001). We have constructed AFP111 *adhE,* AFP111 *poxB* and AFP111 pTrc99A-*glk* as reported in a previous study. The integration of the *pyc* gene from *Rhizobium etli* was studied as described in the Results.

The medium used for microbial growth and succinate production/CO_2 sequestration was developed previously as (per liter): glucose, 40.00 g; citric acid, 3.0 g;; $Na_2HPO_4 \cdot 7H_2O$, 3.00 g, KH_2PO_4, 8.00 g; $(NH_4)_2HPO_4$, 8.00 g; NH_4Cl, 0.20 g; $(NH_4)_2SO_4$, 0.75 g; $MgSO_4 \cdot 7H_2O$, 1.00 g, $CaCl_2 \cdot 2H_2O$, 10.0 mg; $ZnSO_4 \cdot 7H_2O$, 0.5 mg; $CuCl_2 \cdot 2H_2O$, 0.25 mg; $MnSO_4 \cdot H_2O$, 2.5 mg; $CoCl_2 \cdot 6H_2O$, 1.75 mg; H_3BO_3, 0.12 mg; $Al_2(SO_4)_3 \cdot xH_2O$, 1.77 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg; Fe(III) citrate, 16.1 mg; thiamine·HCl, 20 mg; biotin, 2 mg. This medium is *defined*, with its chemical composition completely known and reproducible.

Two-phase fed-batch experiments were carried out in 2.0 L or 2.5 L bioreactors (Bioflow 2000 or Bioflow III, New Brunswick Scientific Co. Edison, NJ, USA) initially containing 1.5 L media. In the growth phase, air and O_2 were mixed at 1.0 L/min total flow rate at 400 rpm constant agitation to maintain a dissolved oxygen concentration (DO) above 40% of saturation. During this aerobic phase, the pH was controlled at a pH of 7.0 using 20% (w/v) NaOH, and the temperature was controlled at 37°C. When the OD reached about 20-25, depending on the specific experiment, the second anaerobic experimental phase was initiated. During this phase, the gas phase composition and conditions of temperature and pH were selected according to the experimental design. Furthermore, several bases to control the pH were compared: 25% NaOH, 25% KOH or 25% $Ca(OH)_2$. In general a feed solution of 40 g/100 mL glucose was added at the initiation of the production phase. Also, in the gas (i.e., containing CO_2) was sparged into fermenter at 0.50 L/min.

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth, and this value was correlated to dry cell mass. The final concentrations of soluble organics compounds were determined by liquid chromatography as previous described (Eiteman and Chastain, 1997).

Pyruvate carboxylase catalyzes the carboxylation of pyruvate to oxaloacetate using ATP and carbonate as co-substrates. The assay used is a coupled enzyme assay (Payne and Morris, 1969) in which the oxaloacetate generated by the action of pyruvate carboxylase reacts with acetyl CoA via citrate synthase (both in excess). The free CoA generated by this second reaction is the species that actually causes the signal by its reaction with the chemical DTNB.

We studied the affect of the gas phase composition using the system shown in Figure 1. Using this set-up, we could readily control the composition of CO_2 and other gases in the entering gas stream. For example, in one set of experiments, we maintained the CO_2 concentration at 50% and the total flow at 0.50 L/min. We were able to study different concentration of NO_2 in the gas phase by selecting the flowrates of the N_2 and the NO_2-in-N_2 gases.

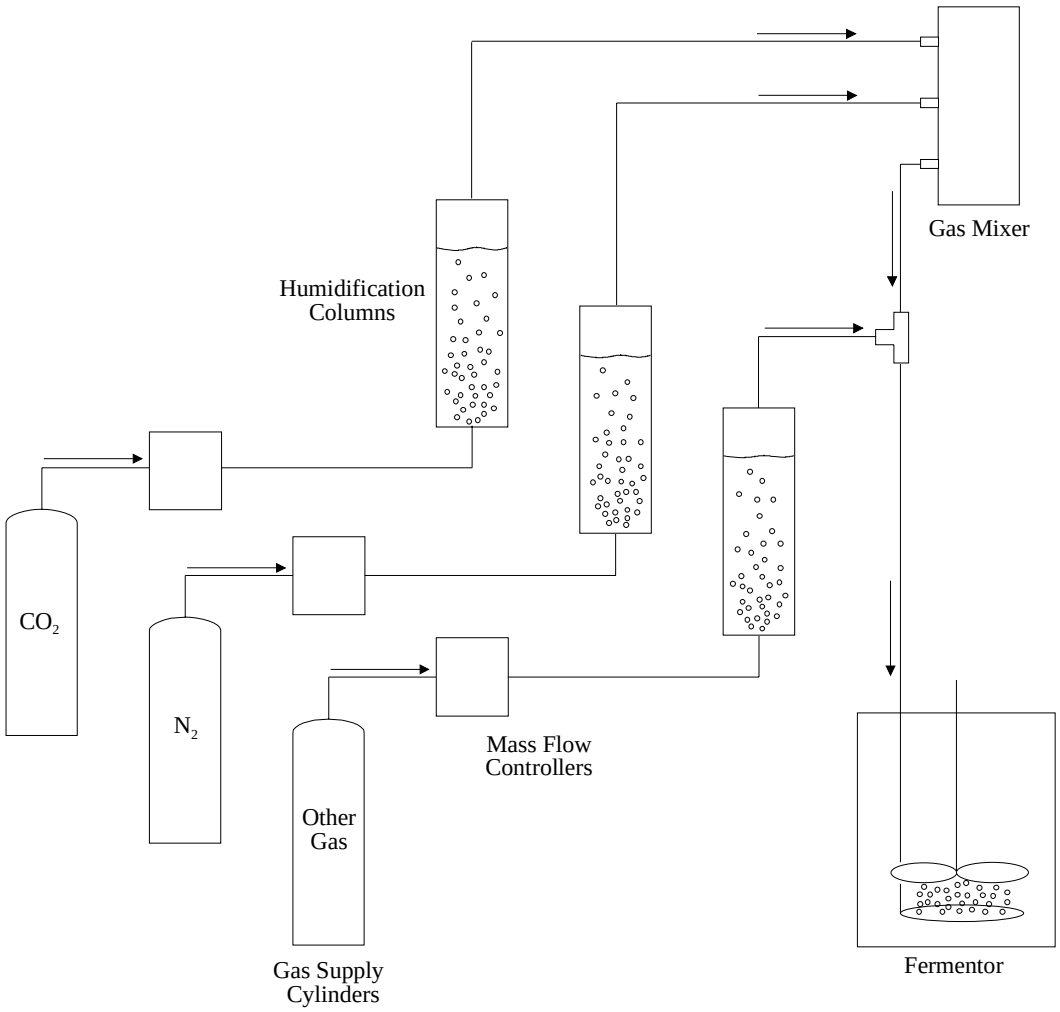

Figure 1: System to determine the effect of gas phase composition on succinate production and CO_2 sequestration.

RESULTS AND DISCUSSION:

1. Strain Development

A significant portion of the effort of this project was devoted to strain development. In particular a variety of molecular biology approaches were tried to integrate the *pyc* gene into the chromosome.

R. etli pyruvate carboxylase was initially cloned into the pTrc99A vector to enable the expression of this anaplerotic enzyme by our laboratory (Gokarn et al., 2001). The pTrc99A-*pyc* construct has been utilized in a variety of metabolic engineering approaches which rely on pyruvate carboxylase, an enzyme that does not naturally occur in wild-type *E. coli* (March et al.,m 2002; Vemuri et al., 2002; Vemuri et al., 2005).

As detailed in the research plan, we had proposed to utilize pTrc99A-*pyc* derivatives we had constructed in which the kanamycin resistance gene from Tn5 had been inserted just downstream of the *pyc* gene to introduce *R. etli pyc* into the chromosome. The *pyc* and kanamycin resistance genes were amplified using the polymerase chain reaction (PCR) and the resulting fragment was recombined into the *E. coli* chromosome using the lambda Red recombination system (Datsenko and Wanner, 2000; Yu et al., 2000). The resulting *E. coli pyc* strain did not express any pyruvate carboxylase activity. This result was not surprising and had been anticipated in the proposal since moving the pTrc99A-*pyc* construct into the chromosome effectively reduced a 200 copy plasmid to a single copy.

To express pyruvate carboxylase at sufficient levels from a single copy of the gene in *E. coli*, we needed a stronger promoter. Two new expression vectors were constructed for this purpose that could accommodate the relatively large 3,500 kb *pyc* gene. The pBR322.P*rrnA* and pBR322.P*ompA* expression vectors were constructed using the strong promoter regions from ribosomal *rrnA* and the outer membrane protein A. Each one of these promoter regions is unusual. The promoter for the *rrnA* gene consists of four distinct promoters which function collectively to form a very strong promoter (deBoer et al., 1979) while the promoter for the *ompA* gene consists of one strong promoter that is operationally linked to four independent Shine-Dalgarno ribosome binding sites (Movva et al., 1980). The *R. etli pyc* gene was cloned into each of these vectors. In the case of the pBR322.P*rrnA* vector, *pyc* was cloned using four different Shine-Dalgarno ribosome binding sites of varying lengths in order to select the clone that expressed maximal amounts of *pyc*. Pyruvate carboxylase activities were determined for all the clones and the clone that expressed the highest levels of pyruvate carboxylase, which was one of the pBR322.P*rrnA*.*pyc* constructs, was selected. The tetracycline resistance gene from Tn10 was cloned into pBR322.P*rrnA*.*pyc* just downstream of the *pyc* gene. This pBR322.P*rrn*A.*pyc* Tet(R) construct was used to PCR amplify a *pyc* Tet(R) cassette to introduce *pyc* into the *E. coli* chromosome.

While the lambda Red recombination system is routinely utilized to insert 1 – 3 kb DNA fragments into the *E. coli* chromosome, the use of this system is problematic for the insertion of larger 4 – 6 kb DNA fragments as required by this research proposal. A cursory examination of the lambda Red recombination vectors that have been developed revealed that the expression of the lambda Beta, Exo and Gamma proteins that constitute the lambda Red recombination system may not be optimal. Based on our laboratories experience with maximizing gene expression in *E. coli*, we decided that an expression vector that maximally expressed the lambda Beta, Exo and Gamma proteins might be developed. Using a couple of different strategies, a pTrc99A derivative that expresses maximal amounts of the lambda Beta, Exo and Gamma proteins was developed. In our preliminary assessment this vector enables recombination to occur at rates that are 10 fold higher than the rates that are achievable by other lambda Red vectors. Our new pTrc99A derivative is also easier to use. To construct a gene knockout or knockin in the final destination strain using vectors developed by other researchers requires 10 days because the initial recombinants must be maintained at 30°C. To construct a gene knockout or knockin using our new pTrc99A derivative which can be maintained at 37°C only requires 4 days.

2. Process Development

During this year of the project, we had several goals in the development of a process for CO_2 sequestration. One on-going problem has been that because we have not yet completed the strain development phase of the research, we still are conducting the experiments using preliminary control strains such as AFP111 rather than this strain with *pyc* integrated on the chromosome (AFP111 *pyc*$^+$). We believe that many, but not all, of the process conditions will be applicable to any of the other strains developed including the production strain. For example, we anticipate that the production strain will behave the same way as this control strain towards NO_2, as one could reasonably expect this gaseous impurity to affect the microbe globally, not just the specific enzyme pyruvate carboxylase. Similarly, we speculate that the production strain will behave only slightly differently from the control strain in its pH optimum. However, because *pyc* is intimately related to CO_2 sequestration, it is more likely that the production strain will behave differently from the control strain in the effect of CO_2 composition. In all cases once the integration of the *pyc* gene on the chromosome is complete, we will have to determine the extent to which this additional sequestration activity impacts process conditions.

During this project period we have focused our process development efforts in three areas, and have made progress in a fourth area. Specifically, we have clarified the range of pH which results in the optimal rate of sequestration. Second, we have determined how the counterion used to control the pH affects the sequestration rate. Third, we have determined how CO_2 gas phase composition impacts sequestration rate. Finally, we have made progress in determining the affect of several potential gaseous impurities on CO_2 sequestration; in particular we have completed a study using NO_2.

Effect of pH

The first goal has been to determine the effect of pH, generally, on CO_2 sequestration. The process has two different phases, and the pH can readily be controlled at different levels during each of the two phases. During the previous project period we concluded that the pH of the system during the initial *growth* phase did not significantly impact the CO_2 sequestration rate during the subsequent phase (within the pH range of 6.2 – 7.0). It should be noted that this previous study used a complex medium rather than the defined medium because we had not yet established a defined medium. However, because we did not see any significant change over the range of pH, we can reasonably conclude that the process is somewhat robust with respect to operational conditions of pH during the initial growth phase. This year our focus has been to study the relationship between pH and CO_2 sequestration during the *production* phase. That is, at what pH is the sequestration rate and succinate production rate the highest?

We conducted these experiments using AFP111 and the defined medium listed in the Experimental section. These experiments were conducted in duplicate in controlled bioreactors at a pH between 5.8 and 7.0, and the results are shown in Table 1 and Figures 2a and 2b. In Table 1 we report our observed succinate production rates and product yields as a function of pH. From this Table several conclusions can be drawn. First, the rate of succinate production is maximal over a relatively small range of pH, between about 6.4 and 6.6. While the difference between this rate was not statistically significant at pH 6.4 and pH 6.6, the rate was 25 % lower

at a pH of 6.2 and 38% lower at a pH of 6.8. Second, the yield of succinate is not a strong function of pH, at least in the pH range of 5.8 – 6.8. Only at a pH of 7.0 was the yield (0.55 g/g) about 24% lower than the maximal yield reported. Third, pyruvate formation as a by-product was greater at lower values of pH. Specially, pyruvate comprised 20% of the mass of the products at a pH of 6.4 or lower, while this by-product was less than 20% of the products at pH values greater than 6.4. Finally, ethanol was always a minor by-product with yields below 0.04, and acetate was significant only at the highest pH of 7.0. The observation that pyruvate formation is greater at lower values of pH is particularly interesting because pyruvate carboxylase uses this compound as a substrate. Thus, one might reasonably speculate that the the flux through the pyruvate carboxylase pathway, and thus the rate of CO_2 sequestration, would be greater at lower values of medium pH. This prediction will have to be determined when the *pyc* gene is integrated on the chromosome. These studies used $Ca(OH)_2$ to control the pH.

The results of our experiments are further presented in Figures 2a and 2b. We are not able to measure CO_2 consumption directly, but based on the stoichiometry of succinate formation, we can calculate the CO_2 utilization rate (Figure 2a). The maximum utilization rate occurs at a pH of 6.4. The formation of an acid in the form of succinic acid, as well as the addition of CO2 into an aqueous environment, together serve to lower the pH over the course of the process. Therefore, base is necessary to control the pH. Obviously, less base is required the control the pH when the set-point pH is more acidic. Figure 2b shows how this fact influences the process. Specifically, an unacceptably large volume of base is needed to neutralize the acid when the pH exceeds 6.6.

Table 1. The effect of pH on succinate volumetric productivity (Q) and specific productivity (q) and mass yield of products (Y) during 14 h of an anaerobic non-growth phase.

pH	Q_S (g/L·h)	q_S (mg/g·h)	Y_S (g/g)	Y_A (g/g)	Y_P (g/g)	Y_E (g/g)
5.8	0.67	76.5	0.71	0.01	0.24	0.00
6.0	1.02	126.0	0.67	0.01	0.21	0.02
6.2	1.18	125.7	0.65	0.02	0.24	0.02
6.4	1.59	195.3	0.65	0.00	0.24	0.04
6.6	1.49	169.3	0.66	0.00	0.16	0.03
6.8	0.99	119.4	0.72	0.01	0.19	0.03
7.0	0.36	47.4	0.55	0.06	0.05	0.02

Subscripts: S: succinate, A: acetate, P: pyruvate, E: ethanol

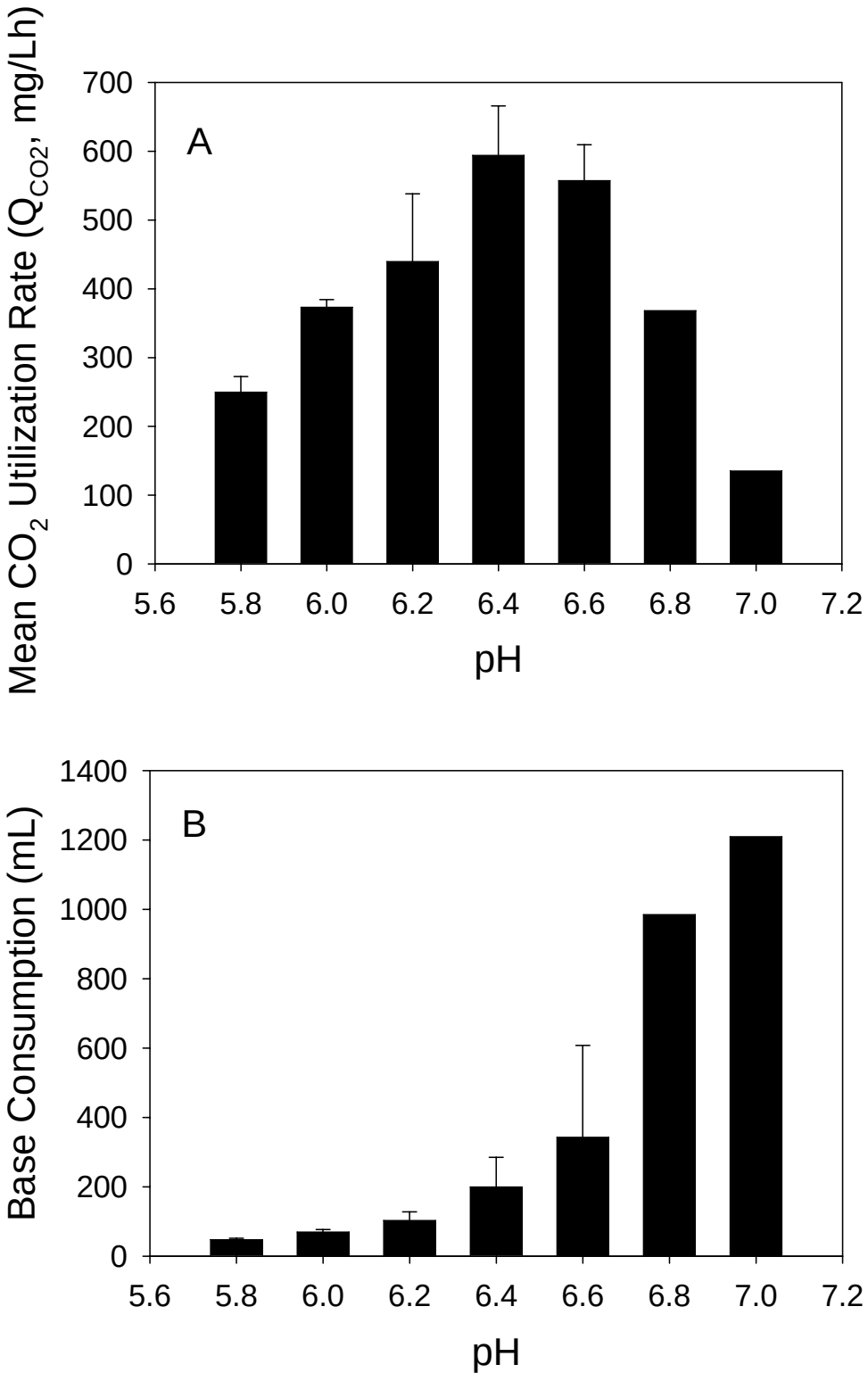

Figure 2. Effect of pH on A) mean CO_2 utilization rate and B) volume of 25% $Ca(OH)_2$ consumed during 14 h of an anaerobic non-growth production phase.

Based on the results shown in these figures, we conclude that the process for CO_2 sequestration will operate optimally near a pH of 6.4. The maximum rate observed was about 600 mg/Lh, which is approximately 46 times the rate observed by other researchers who used a photobioreactor. There are several cautionary comments related to this conclusion. First, as noted elsewhere, we base this conclusion on the control strain AFP111 rather than the production strain AFP111 *pyc*+. Additional experimentation will be required to determine whether this one enzyme alters the optimal pH. Second, we have not determined how other phenomenon might be influenced by pH (e.g., NO_2 inhibition). Finally, these experiments were conducted using a gas phase composition of 100% CO_2. A lower composition of CO_2 may affect the value of the maximal rate, but would probably not change pH ot which this maximal rate occurs. A lower composition of CO_2 would likely result in lower base consumption.

Effect of Base Counterion

We demonstrated that a pH of 6.4 resulted in the maximum rate of CO_2 sequestration. Several bases could be used to control that pH. In our experiment described previously in this report we used 25% $Ca(OH)_2$. Other inexpensive bases include KOH and NaOH. We completed a simple set of experiments to determine which of these three bases resulted in the best succinate production/CO_2 sequestration at the optimal pH of 6.4. We presume in this study that the optimal pH when using one base corresponds with the optimal pH when using another base. Table 2 summarizes the results.

As described previously, the mean CO_2 utilization rate (Q_{CO2}) was nearly 600 mg/Lh when $Ca(OH)_2$ was used to control the pH at 6.4. For both KOH and NaOH, the mean CO_2 utilization rate was about 40% below that value. Moreover, the rate of CO_2 utilization continued to decrease substantially with time for KOH and NaOH, but this rate was stable for $Ca(OH)_2$. We speculate that $Ca(OH)_2$ does not deleteriously impact CO_2 utilization because calcium succinate is not greatly soluble in aqueous solutions. Essentially the process involves the conversion of gaseous CO_2 partially into a solid product. This phenomenon is advantageous because it suggests that theCO_2 sequestration can be prolonged much further with $Ca(OH)_2$ before the cells have to be "rejuvenated".

Table 2. The effect of base counterion on succinate volumetric productivity (Q_S), specific productivity (q_S), volumetric CO_2 utilization rate (Q_{CO2}), specific CO_2 utilization rate (q_{CO2}), and mass yield (Y_S) during 14 h of an anaerobic non-growth phase at a controlled pH of 6.4.

Base (25% w/v)	Q_S (g/L·h)	q_S (mg/g·h)	Q_{CO2} (mg/L·h)	q_{CO2} (mg/g·h)	Y_S (g/g)
$Ca(OH)_2$	1.59 (0.19)	195 (24)	594 (72)	72 (9)	0.66 (0.14)
KOH	0.88 (0.07)	95 (6)	328 (25)	36 (2)	0.54 (0.08)
NaOH	0.99 (0.19)	102 (24)	367 (69)	38 (9)	0.57 (0.04)

Data in parentheses were standard deviation from duplicated experiments

Effect of gas phase CO_2 composition

The results reported previously in this document were conducted using a gas phase composition of 100% CO_2. Obviously, many readily available gas streams such as flue gas do not contain 100% CO_2, but contain a lower concentration of this gas mixed with N_2 and potentially many other gases at lower concentrations. Therefore, we conducted a series of experiments in which the gas phase concentration of CO_2 was set at one of several different levels, with the balance of the gas being composed of N_2. The results of these experiments are shown in Table 3 and Figure 3. The results are striking. The gas phase composition does not have an effect on succinate yield, but it does have an effect on rates of succinate production and CO_2 utilization. The effect is essentially linear, with increasing concentrations of CO_2 resulting in a proportionate increase in CO_2 utilization. This result has a couple interesting interpretations. First, if the CO_2 utilization is proportional to the gas phase CO_2 concentration, or partial pressure, then increasing the pressure beyond atmospheric conditions should further increase the CO_2 utilization rate. For example, the partial pressure of CO_2 will be identical when 100% CO_2 is at 1 atmosphere as when 10% CO_2 is at 10 atmosphere. We are not able to examine whether this prediction is true because our bioreactors cannot sustain a pressure significantly greater than atmospheric conditions. However, it would be quite plausible to operate a CO_2 sequestration process at substantially higher pressure than atmospheric. The second interesting interpretation is that if CO_2 utilization is increasing with concentration, then CO_2 concentration should be limiting CO_2 utilization.

Table 3. The effect of gas phase CO_2 composition on succinate volumetric productivity (Q_S), specific productivity (q_S), volumetric CO_2 utilization rate (Q_{CO2}), specific CO_2 utilization rate (q_{CO2}), and mass yield (Y_S) during 14 h of an anaerobic non-growth phase at a controlled pH of 6.4.

% CO2	Q_S (g/L·h)	q_S (mg/g·h)	Q_{CO2} (mg/L·h)	q_{CO2} (mg/g·h)	Y_S (g/g)
10	0.25 (0.08)	29 (24)	594 (72)	72 (9)	0.51 (0.37)
20	0.52 (0.10)	58 (12)	193 (37)	22 (5)	0.50 (0.08)
50	0.76 (0.07)	95 (9)	282 (26)	36 (3)	0.47 (0.08)
100	1.48 (0.35)	182 (44)	552 (131)	68 (16)	0.65 (0.16)

Data in parentheses were standard deviation from duplicated experiments

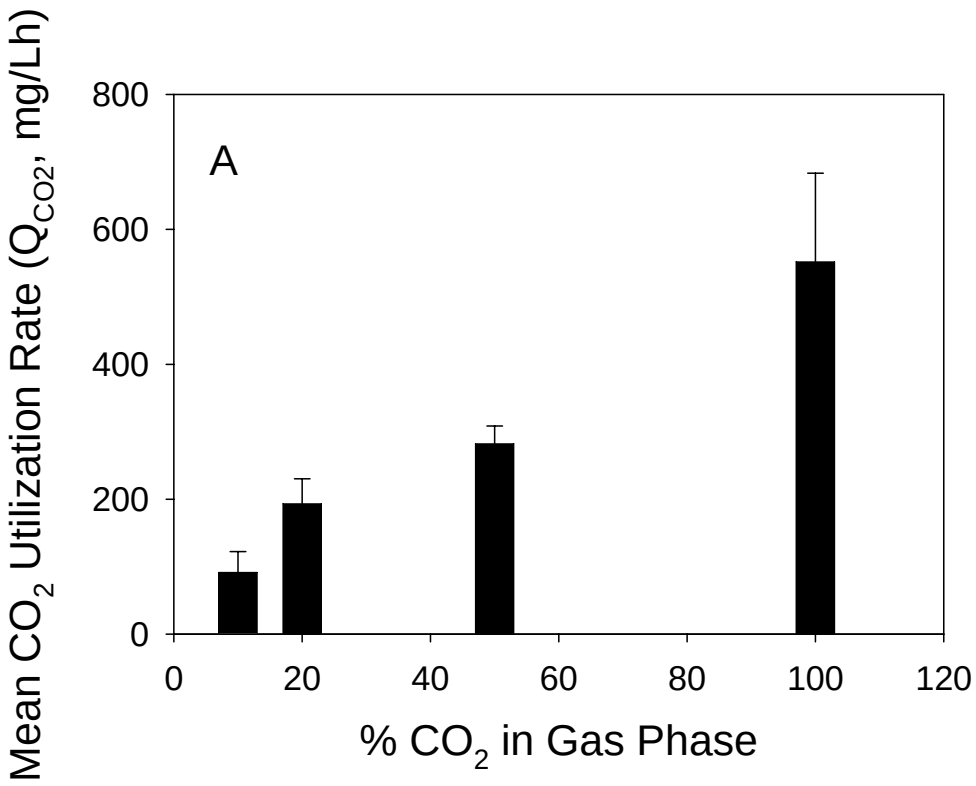

Figure 3. Effect of gas phase composition of CO_2 on CO_2 utilization rate during 14 h of an anaerobic non-growth production phase.

We completed calculations to try to understand what could be limiting CO_2 utilization. The particular strain we studied was AFP111, the control strain in which CO_2 sequestration occurs exclusively by the action of the enzyme PEP carboxylase. For this enzyme the bicarbonate ion, HCO_3^-, is essential for PEP carboxylase to sequester CO_2. Does the rate of bicarbonate formation limit the action of this enzyme? The formation of succinate (and utilization of CO2) is related to four sequential rates shown in Figure 4. At steady-state, each of the rates must be equal. During the process which used 50% CO_2 delivered to the bioreactor, for example, we measured the rate of CO_2 incorporation into succinate (R) at 282 mg/L·h (from Table 3) or at a specific rate of 36 mg/g·h.

First, CO_2 must be introduced into the fermentor by sparging a gas containing CO_2, and CO_2 transfers from the gas bubbles to the liquid. The rate of CO_2 transfer from the gas to liquid phase is given by:

$$\frac{d[CO_2]}{dt} = k_L a\left([CO_2]_{eq} - [CO_2]\right) = \Delta CO_2$$

where $k_L a$ is the volumetric CO_2 mass transfer coefficient (h^{-1}), $[CO_2]_{eq}$ is the molar CO_2 concentration in equilibrium with the gas phase, and $[CO_2]$ is the liquid phase (bulk) molar CO_2

concentration. We measured the mass transfer coefficient for oxygen in our system at conditions identical to those used during the CO_2 sequestration phase (e.g., 200 rpm). From this experiment we estimated the value of $k_L a$ for CO_2 to be 28.9 h^{-1}. Therefore, the driving force for mass transfer ΔCO_2 is about 9 mg/L, or 0.20 mM. Since the value of $[CO_2]_{eq}$ at 37°C using 50% CO_2 is 13 mM, the value of $[CO_2]$ must essentially equal $[CO_2]_{eq}$, and the process of dissolving CO_2 into the fermenter is not mass transfer limited.

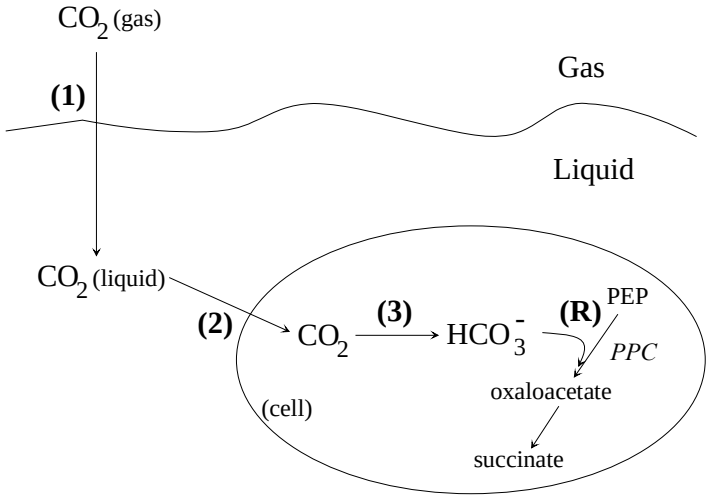

Figure 4 Model of CO_2 utilization

The second rate process is the diffusion of CO_2 from the bulk liquid to the surface of a cell. The diffusion equation for a single elongated cell is given by (Berg, 1983a):

$$I = \frac{4\pi D_{GAS} a[CO_2]}{ln(2a/b)}$$

In this equation, D_{GAS} is CO_2 diffusion coefficient (m^2/s) and I is molar flux of CO_2 (i.e., mol/cell·s). The typical size of an *E. coli* cell is 2×10^{-4} cm (a) by 10^{-4} cm (b) (Berg, 1983b). The value for D_{GAS} at 20°C is 1.78×10^{-9} m^2/s. Using an average cell dry weight of 2.8×10^{-13} g (Merlin et al., 2003), then the maximum rate of diffusion is 1.5 mol/g·s or 2.4×10^{8} mg/g·h, about 7 orders of magnitude greater than the actual rate observed (i.e., 36 mg/g·h). In other words, at the concentration of CO_2 in the bulk the rate of reaction is far less than the possible rate of diffusion. Therefore, the transport of CO_2 into the cell is not limited by the mass transfer of CO_2 from the bulk to the surface of the cell. Moreover, one concludes that the surface concentration of CO_2 is equal to the concentration in the bulk, or about 13 mM.

The third rate process is the transport of CO_2 across the cell membrane. Recent results indicate that red blood cell membranes provide negligible resistance to mass transport. We have no way to measure trivially in our system the resistance of the membranes to mass transport. However, if like the red blood cells the resistance is negligible, then the internal concentration of CO_2 is approximately equal to the external surface CO_2 concentration, about 13 mM.

The fourth rate process is the conversion of dissolved CO_2 intracellularly to bicarbonate following:

$$CO_2 + H_2O \leftrightarrows HCO_3^- + H^+ \quad (1)$$

This process is usually described by two processes:

$$CO_2 + H_2O \rightarrow H_2CO_3 \quad (2)$$

$$H_2CO_3 \rightarrow HCO_3^- + H^+ \quad (3)$$

The first reaction (eqn. 2) has a forward first order rate constant of 0.03 s^{-1} (Stumm and Morgan, 1996). Therefore the rate of this reaction using a CO_2 concentration of 13 mM is 62000 mg/L·h, about 200 times greater than the rate of CO_2 incorporation we observed (282 mg/L·h). This result means that this reaction will essentially be at equilibrium. The equilibrium constant for eqn. 1 is $10^{-6.381}$ (Rubio et al. 1999). Using similar arguments for the second reaction (eqn. 3), we calculate that the rate of reaction far exceeds the rate of CO_2 incorporation we observed. So, the concentration of HCO_3^- should be in equilibrium with the concentration of CO2. Thus, the bicarbonate concentration at a pH of 6.4 should be 15 mM. If we assume the intracellular pH is maintained at 7.0 (more realistic), then this value should be 58 mM. In either case, this concentration far exceeds the Michaelis constant for *E. coli* PEP carboxylase (0.1 mM). This result suggests that the reaction catalyzed by PEP carboxylase is saturated and operating at its maximum rate.

To summarize, our calculations indicate that an increase of CO_2 concentration in gas phase would not cause an increase in the rate of CO_2 sequestration by PEP carboxylase. However, our observations show that we do indeed observe an increase in the rate of CO_2 sequestration with an increase in CO_2 concentration. We cannot offer an explanation for the observations and are presently trying to understand and rationalize our results.

Effect of impurities in the gas phase

As noted elsewhere, industrial gas streams exist which contain impurities in addition to CO_2. We wanted to assess the robustness of this process toward a variety of gaseous components to learn of its applicability using real streams. Impurities that can be found in flue gas, for example, include NO_2, SO_2, CO and O_2. We first examine NO_2, which as a result of its high human toxicity, we believed to represent a "worst-case" situation for the proposed biological process. Because of the toxicity of several of these gases, substantial safety equipment and procedures had to be incorporated into the plan.

We began to examine the effect of NO_2 on CO_2 utilization using 50% CO_2 in the gas phase. These values can be compared with the baseline case of 50% CO_2 in the absence of NO_2 (as observed in Table 3). Table 4 and Figure 5 show the results. Although the standard errors are fairly large, concentrations of NO_2 as high as 200 ppm do not have a negative effect on CO_2 utilization with this process. Indeed, the presence of NO_2 appears to slightly improve CO_2 sequestration. Although we do not know why NO2 would result in an improvement in CO_2 utilization, we speculate that the cause for this observation is due to the oxidizing effect of NO_2. Because the direct conversion of glucose to succinate via PEP carboxylase (and pyruvate

carboxylase) is not redox balance, having a slightly oxidizing environment may help drive the process. In any event the conclusion is that NO_2 present in the gas phase, at least up to 200 ppm, would not negatively effect CO_2 utilization.

Table 4. The effect of NO_2 in the gas phase on succinate volumetric productivity (Q_S), specific productivity (q_S), volumetric CO_2 utilization rate (Q_{CO2}), specific CO_2 utilization rate (q_{CO2}), and mass yield (Y_S) during 14 h of an anaerobic non-growth phase at a controlled pH of 6.4. The gas phase contained 50% CO_2.

NO_2 (ppm)	Q_S (g/L·h)	q_S (mg/g·h)	Q_{CO2} (mg/L·h)	q_{CO2} (mg/g·h)	Y_S (g/g)
0	0.76 (0.07)	95 (9)	282 (26)	36 (3)	0.47 (0.08)
50	1.09 (0.34)	121 (24)	405 (128)	45 (9)	0.54 (0.00)
200	1.20 (0.44)	147 (76)	447 (165)	55 (29)	0.63 (0.06)

Data in parentheses were standard deviation from duplicated experiments

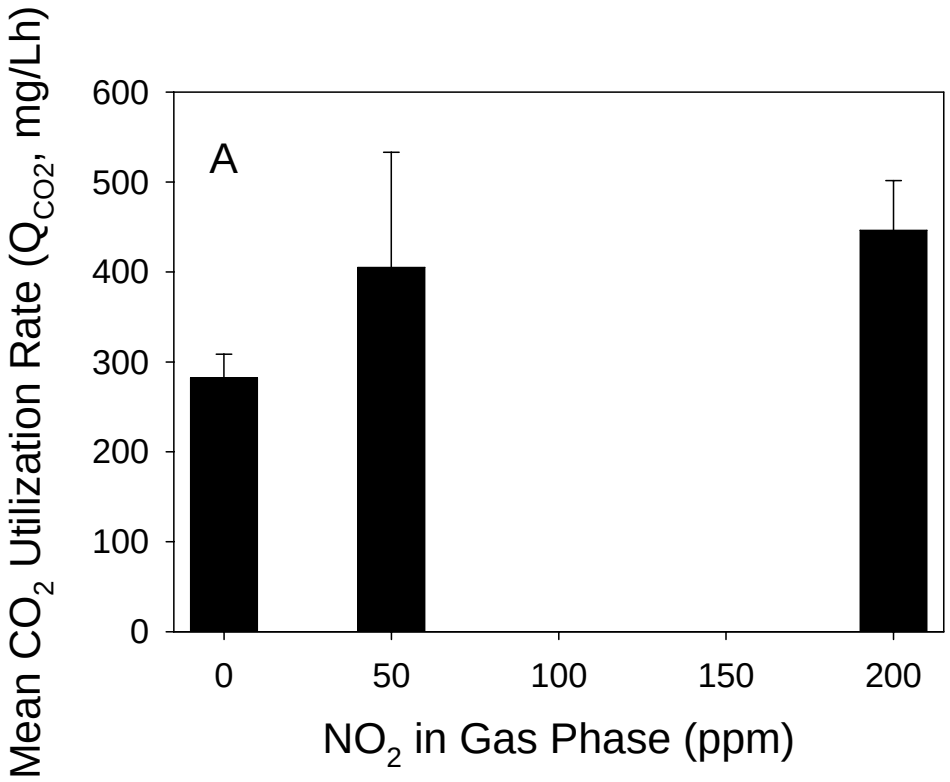

Figure 5. Effect of NO_2 in the gas phase on CO_2 utilization rate during 14 h of an anaerobic non-growth production phase. The gas phase contained 50% CO_2.

CONCLUSIONS:

We are progressing with the integration of the *pyc* gene from *Rhizobium etli* onto the chromosome of our production strain *E. coli* AFP111. This component of the research has proved very challenging because of the need for strong promoters and because of the size of the *pyc* gene.

We have determined that the optimal pH for CO_2 sequestration using AFP111 is about 6.4. The rates were determined using $Ca(OH)_2$ as base and 100% CO_2. We expect that other bases, gas phase concentrations or the production strain AFP111 *pyc+* will not significantly alter this optimal pH.

We have determined that $Ca(OH)_2$ is the preferred base to use to control the pH. We speculate that this result is due to the comparative insolubility of calcium succinate, which minimizes the amount of dissolved counterion in the production medium. It is possible that a process could be developed to separate this solid material from the microbes in a continuous fashion. This result was determined using 100% CO_2 at a pH of 6.4.

We have determined that the concentration of CO_2 in the gas phase has a linear relationship with CO_2 sequestration. Considering the various rate phenomena which occur in this system and the kinetics of the single enzyme that provides carbon fixation, we are not able to explain why this result has occurred. The presence of pyruvate carboxylase activity, an additional enzyme for CO_2 sequestration, might further increase the rate of CO_2 utilization. Also, the result suggests that a further increase in CO_2 concentration—for example, but pressurizing the system—would be a means to increase the rate of CO_2 sequestration.

We have demonstrated that NO_2 in the gas phase actually enhances the CO2 sequestration slightly, at least up to a concentration of 200 ppm. We rationalize this intriguing result by considering the oxidizing effect dilute NO_2 would have on our system which might serve to overcome a slight the redox imbalance in the microbial process. We continue to study the reason for this observation as it might lead to a further improvement in the process.

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